2019

Metabolic Engineering for Biocatalyst Robustness to Organic Inhibitors

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Abstract
Microbial production of biorenewable fuels and chemicals is often limited by inhibition of the biocatalyst, either by increasing concentrations of the product compound or by contaminant compounds in the biomass-derived sugars. This inhibition can interfere with economically viable production. Here we discuss typical mechanisms of inhibition and methods for improving biocatalyst robustness. Inhibition often takes the form of inhibition of enzyme activity, depletion of cofactor pools, and membrane damage; methods are discussed for mitigating each of these types of inhibition. Various evolutionary schemes have been developed and implemented on a variety of inhibitory compounds, including butanol, acetic acid, furfural, and ethanol. Reverse engineering of these improved strains can provide insight into new metabolic engineering strategies.

Keywords
metabolic engineering, metabolic process engineering, bacteria, biocatalysis, microbial physiology

Comments
This is a chapter from Royce, Liam and Laura R. Jarboe. "Metabolic Engineering for Biocatalyst Robustness to Organic Inhibitors." In *Bioprocessing Technology for Production of Biopharmaceuticals and Bioproducts*, First Edition. Edited by Claire Komives and Weichang Zhou. Hoboken, NJ: John Wiley & Sons, Inc., 2019: 241-265. DOI: 10.1002/9781119378341.ch7. Posted with permission.

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Metabolic Engineering for Biocatalyst Robustness to Organic Inhibitors

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7.1 Introduction

The field of metabolic engineering has grown enormously since its definition in 1991 (Bailey 1991; Stephanopoulos and Vallino 1991). Since then, enormous progress has been made in the use of engineered microbes for production of biorenewable fuels and chemicals. Two years after its initial definition, Cameron and Tong classified five types of metabolic engineering: enhanced production of native metabolites; heterologous production of foreign metabolites; utilization of new substrates for metabolism; improved or new metabolic pathways for chemical degradation; and modification of cell properties that facilitate bioprocessing (Cameron and Tong 1993). Many landmark metabolic engineering projects have dealt with the first three strategies, including but not limited to (Ohta et al. 1991; Zhang et al. 1995; Steinbuchel 2001; Nakamura and Whited 2003; Jeffries and Jin 2004; Lutke-Eversloh and Stephanopoulos 2007; Atsumi et al. 2008), and can be thought of as relating mainly to metabolic functionality. Here we focus on the modification of cell properties that facilitate bioprocessing. Such modifications have focused on a wide variety of biocatalyst properties; here we focus on the property of biocatalyst robustness, particularly in regards to organic inhibitor tolerance. This improved robustness can be geared toward increasing product tolerance, possibly enabling increased product titers, and increased ability to use "dirty" biomass-derived sugars.

While industrial (Chotani et al. 2000) and academic researchers (Zeng and Biebl 2002; Demain 2006; Jarboe et al. 2007; Yan and Liao 2009) have reported attainment of high titers, yields, and productivities for some products, this is something that remains difficult to achieve, especially with next-generation biofuels and biochemicals. High product titers are desirable

Bioprocessing Technology for Production of Biopharmaceuticals and Bioproducts, First Edition.
Edited by Claire Komives and Weichang Zhou.

to make downstream separation steps cost-effective, but can be toxic to the microbial biocatalyst. Biocatalyst inhibition is also problematic when biomass-derived sugars are used as the fermentation substrate, as biomass hydrolysate or pyrolysate ("dirty sugars") contains trace contaminants that are inhibitory to the microbe (Mills et al. 2009; Lian et al. 2010; Jarboe et al. 2011a). This toxicity means that the amount of sugars that can be utilized by the organism is limited, and thus the amount of product formed is decreased relative to fermentations using "clean" sugars. One approach for dealing with this product or feedstock toxicity is to remove the inhibitory compounds (Martinez et al. 2000; Lennen et al. 2010; Lian et al. 2010; Lakshmanaswamy et al. 2011; Chi et al. 2013). However, here we focus on the complementary approach of improving microbial robustness to organic inhibitors. Note that there are also reports of inhibitory side products; these can often be addressed by re-distribution of the metabolic flux (Agrawal et al. 2012).

Biocatalyst robustness as it relates to the economically viable production of fuels and chemicals has been discussed in a variety of recent reviews (Warnecke and Gill 2005; Fischer et al. 2008; Mills et al. 2009; Nicolaou et al. 2010; Dunlop 2011; Jarboe et al. 2011b). In this chapter, we highlight some of the key concepts in this field and cover some of the recent findings. The discussion includes metabolic evolution coupled with reverse engineering, a valuable tool for gaining insight into the mechanism of inhibition and strategies for increasing tolerance. Figure 7.1 highlights the possible mechanisms of inhibition by organic compounds, while Table 7.1 lists successful strategies to increase tolerance. Most of our discussion involves either *Escherichia coli* or *Saccharomyces cerevisiae*, since they are the two most commonly used organisms both for metabolic engineering and for understanding tolerance.

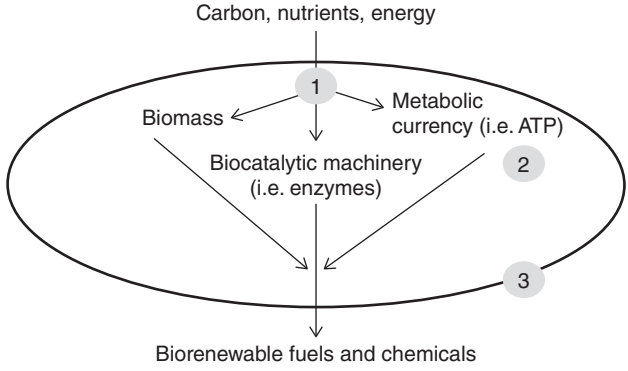


Figure 7.1 Reported mechanisms of inhibition often take the form of (1) inhibition reactions that produce metabolic precursors, limiting the formation of biomass and/or the enzymes needed for metabolism; (2) depletion of general metabolic precursors, such as ATP and NADPH; (3) damage to the cell membrane, interfering with membrane-associated reactions, and retention of valuable metabolites.

Table 7.1 Common types of microbial inhibition by organic inhibitors.

General mechanism	Example	Source
Inhibition of production of essential metabolites	Depletion of NADPH by aldehyde reduction limits production of cysteine	Miller et al. (2009a)
	Inhibition of methionine biosynthesis during acetate challenge	Whitfield et al. (1970), Roe et al. (2002)
	Inhibition of isoleucine and leucine biosynthesis during valine challenge	Manten and Rowley (1953)
	Inhibition of chorismate and threonine production by 3-HP	Warnecke et al. (2010)
Membrane damage	Membrane leakage induced during carboxylic acid challenge and production	Zaldivar and Ingram (1999), Lennen and Pfleger (2013), Liu et al. (2013), Royce et al. (2013)
	Reduced function of membrane-associated processes during carboxylic acid challenge	Teixeira et al. (2004), Cipak et al. (2008), Ruenwai et al. (2011)
Intracellular acidification	Low intracellular pH during acetate challenge	Trcek et al. (2015)

7.2 Mechanisms of Inhibition

The mechanism by which different compounds limit biocatalyst growth and metabolism vary according to the chemistry of the molecule. Discussed in this section are inhibition of production of essential metabolites, such as amino acids and nucleotides, membrane damage, and perturbation of intracellular pH (Table 7.1), but it should be noted that there are a variety of known pathogenesis-related inhibition mechanisms, such as antibiotics and reactive oxygen or reactive nitrogen species (Wright 2003; Dzidic et al. 2008; De Pascale and Wright 2010). Omics analysis, such as transcriptome analysis, has often played a key role in identification of these mechanisms of inhibition, as reviewed elsewhere (Jarboe et al. 2011b).

Given the necessity of amino acids and nucleotides for production of proteins, transcripts, and genome replication, disturbance of their production can lead to biocatalyst inhibition. This can be especially problematic when the desired growth condition is defined minimal media, relative to metabolite-containing rich media. Depletion of essential metabolic building blocks can trigger the global stringent response (Jain et al. 2006; Durfee et al. 2008). For example, it was shown that the biomass-derived inhibitor furfural

indirectly inhibits cysteine production in *E. coli* by depleting the NADPH needed for conversion of sulfate to hydrogen sulfide (Miller et al. 2009a). The accumulation of carboxylate ions in *E. coli*, such as during challenge with carboxylic acids, can increase the intracellular ionic strength to levels sufficient to inhibit homocysteine transmethylase (MetE), an enzyme required for methionine biosynthesis (Whitfield et al. 1970; Roe et al. 2002). The feedback loop that regulates valine production in *E. coli* results in valine-mediated inhibition of production of leucine and isoleucine. Thus, valine can be inhibitory to some *E. coli* strains when leucine and isoleucine are not exogenously supplied (Manten and Rowley 1953). 3-Hydroxypropionic acid (3-HP) can limit amino acid synthesis via inhibition of the chorismate and threonine superpathway (Warnecke et al. 2010). This limitation of biosynthesis pathways is distinct from situations where the product compound inhibits its own production; enzyme improvement for alleviating this problem has been reviewed elsewhere (Jarboe et al. 2012).

Another commonly noted mechanism of inhibition is membrane damage. This is especially problematic when dealing with hydrophobic compounds, such as carboxylic acids and butanol (Lennen et al. 2011; Jarboe et al. 2013; Liu et al. 2013; Royce et al. 2013). Overton's Rule provides a general rule of thumb for predicting the membrane permeability of a compound, whereby compounds with increased lipid solubility (i.e. hydrophobicity) have greater membrane permeability (Al-Awqati 1999). Membrane damage can manifest as a failure to maintain appropriate fluidity and/or leakage of valuable metabolites. Leakage is often monitored using Mg^{2+} as a reporter molecule (Zaldivar and Ingram 1999; Liu et al. 2013; Royce et al. 2013) or permeability to nucleic acid stains (Lennen and Pfleger 2013). Fluidity can be measured via the diffusivity of a fluorescent reporter molecule (Beney et al. 2004; Mykytczuk et al. 2007). Recent studies have visualized membrane pores, and eventual disintegration, caused by an antimicrobial peptide (Rakowska et al. 2013), though such studies have not yet been performed with the type of inhibitors of interest here. Membrane damage can result in improper functioning of membrane-associated reactions, such as the electron transport chain, sometimes resulting in increased production of reactive oxygen species (Teixeira et al. 2004; Cipak et al. 2008; Ruenwai et al. 2011).

Exogenous challenge with carboxylic acids, especially acetate, has been shown to disrupt intracellular pH (Viegas et al. 1998; Ricke 2003; Royce et al. 2014; Trcek et al. 2015). This drop in intracellular pH is due to dissociation of the carboxylic acid in the cell interior. The acidification can inhibit cellular processes and impose a hefty ATP burden as ATPase is used to remove the excess protons (Viegas et al. 1998).

Thus, biocatalyst inhibition can occur at a variety of levels and scales. Inhibition of a single biosynthesis-related enzyme can completely inhibit biosynthesis of the machinery needed for growth and metabolism. An increase in the

harshness of the intracellular environment, such as increased osmotic stress or a drop in pH can stress multiple enzymes. Depletion of generic cofactors, such as NAD(P)H and ATP, can have a general slowing effect on metabolism. Membrane damage can impact membrane-associated processes and result in leakage of valuable metabolites. A variety of metabolic engineering strategies have been demonstrated for dealing with these various types of inhibition.

7.3 Mechanisms of Tolerance

Strategies for increasing biocatalyst robustness of organic inhibitors have arisen both as a deliberate response to known mechanisms of inhibition and from analysis of evolved strains. Here we highlight a few of these known mechanisms (Table 7.2), while evolutionary strategies to increase tolerance are described later in this chapter.

A design scheme that has arisen somewhat independently of known inhibition mechanisms or analysis of evolved strains is the provision of appropriate transporter proteins. This strategy asserts that product toxicity can be alleviated, at least in part, by providing cells with the opportunity to expel the problematic compound. This strategy has proven effective in improving both tolerance and production of limonene (Dunlop et al. 2011) and valine (Park et al. 2007) by *E. coli*, as well as alkane tolerance in *S. cerevisiae* (Ling et al. 2013), geraniol tolerance in *E. coli* (Shah et al. 2013) and decanoate tolerance in *E. coli* (Lennen et al. 2013).

Problems with biosynthesis due to inhibition of enzyme activity can be addressed by replacing the sensitive enzyme with an isozyme or mutant enzyme that is resistant to the inhibitory effect. This approach has been successfully demonstrated with both valine and 3-HP (Warnecke et al. 2010; Park et al. 2011). Depletion of generic cofactors, such as furfural-mediated depletion of NADPH, can be addressed by either deleting the enzyme responsible for this depletion (Miller et al. 2009b) or implementing metabolic changes to increase cofactor availability (Wang et al. 2011, 2013; Zheng et al. 2013). For instance, provision of an NADH-dependent furfural reductase enabled *E. coli* to reduce furfural to the less toxic furfuryl alcohol, while not depleting the NADPH needed for biosynthesis (Wang et al. 2011; Zheng et al. 2013).

Acetate and other organic acids are known to decrease the intracellular pH of microorganisms, as previously discussed. The balance between extracellular and intracellular pH greatly affects the proton motive force (PMF), which is important for ATP production and transport processes. *E. coli* can adapt to changes in pH by accommodating the electrochemical potential (the balance of chemical charges) in order to maintain the appropriate PMF (Royce et al. 2014). Other adaptations of organic acid tolerance include addition of cyclopropane fatty acids in the cell membrane, as discussed below.

Table 7.2 Common methods for improving tolerance.

Method	Example	Source
Provision of exporters for the inhibitory compound	Production of limonene	Dunlop et al. (2011)
	Production of valine	Park et al. (2007)
	Alkane tolerance	Ling et al. (2013)
Membrane engineering	Alteration of the distribution of saturated and unsaturated fatty acids improved ethanol tolerance	Luo et al. (2009)
	Alteration of the distribution of saturated and unsaturated fatty acids improved carboxylic acid tolerance	Lennen and Pfleger (2013)
Directed evolution	Improved isobutanol tolerance	Atsumi et al. (2010), Minty et al. (2011)
	Improved tolerance of biomass hydrolysate	Huang et al. (2009), Mills et al. (2009), Geddes et al. (2011)
Enrichment of expression libraries	multi-SCalar Analysis of Library Enrichments (SCALEs)	Gall et al. (2008), Warnecke et al. (2010, 2012), Sandoval et al. (2011), Woodruff et al. (2013)
	Global transcription machinery engineering (gTME)	Alper and Stephanopoulos (2007, Pan et al. (2009), Zhang et al. (2010), Chen et al. (2011), Liu et al. (2011), Ma et al. (2011), Yang et al. (2011), Lanza and Alper (2012), Ma and Yu (2012), Wang et al. (2012), Chong et al. (2013)
Combinatorial expression of protective genes	Ethanol tolerance	Nicolaou et al. (2012)

As mentioned above, membrane damage is a frequently cited mechanism of inhibition and membrane-associated genes have been identified in the analysis of many strains evolved for various tolerance phenotypes (Sandoval et al. 2011; Woodruff et al. 2013). For this reason, we have designated a distinct section for describing membrane engineering efforts.

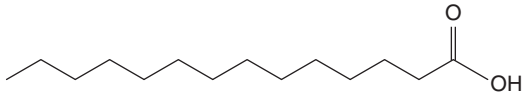
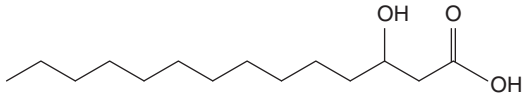
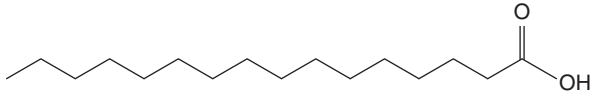
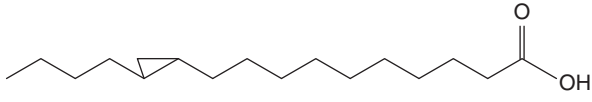
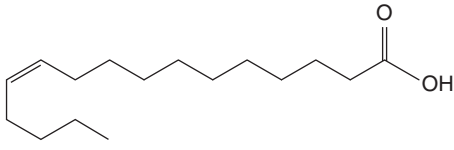
7.4 Membrane Engineering

Modulation of the membrane composition enables microbes to respond to environmental challenges (Zhang and Rock 2008). The lipid-rich cell membrane is often targeted at the molecular level by hydrophobic compounds. As described above, this can result in decreased ability of the membrane to

retain valuable metabolites and decreased function of membrane-associated metabolic processes. For this reason, many of the most interesting recent breakthroughs in metabolic engineering for inhibitor tolerance are related to membrane engineering. We describe these results here, after a brief summary of the physiological role of the different bacterial membrane components.

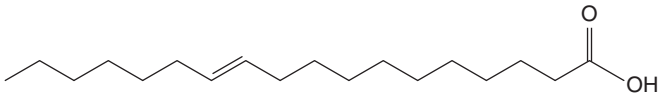
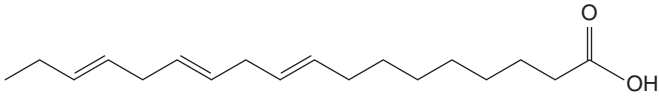
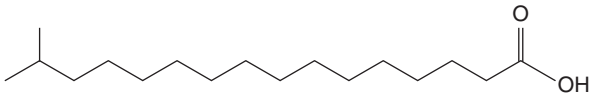
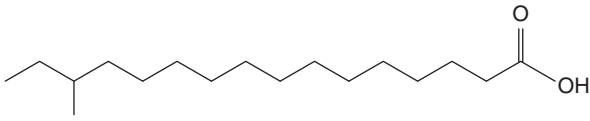
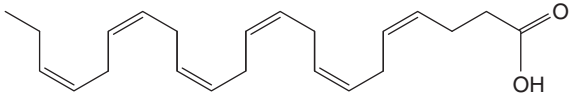
Bacterial membranes are a mixture of many different lipid entities (Table 7.3). Each lipid species plays a distinct physiological role. The main lipid component of the *E. coli* membrane is the straight-chain, unsaturated palmitic acid (C16:0).

Table 7.3 Physiological significance of selected types of bacterial membrane lipids.

 C14:0 *not shown: C12:0, C18:0*	*E. coli* membranes contain minor amounts of these lipids. *E. coli* incorporates C14:0 into lipid A (Raetz et al. 2007). Fluidity is dependent on chain length and degree of saturation; short lipids increase fluidity and long lipids decrease fluidity
 3-hydroxy-C14:0	C12:0 or C14:0 fatty acids bind to the hydroxyl group of 3-hydroxy-C14:0 in lipid A of *E. coli*
 C16:0 (palmitic acid)	*E. coli* bacteria membranes are predominantly C16:0
 Cyclopropane C17cyc *not shown: C19cyc*	Gram-negative bacteria incorporate more cyclopropane fatty acids during stationary phase as a stress response (Chang and Cronan 1999). C17cyc helps mediate acid stress
 Monounsaturated *cis*-C16:1 *not shown: C18:1*	An increase in unsaturated lipids increases membrane fluidity

(Continued)

Table 7.3 (Continued)

 *trans* C18:1	Trans fatty acids occur naturally in *Pseudomonas putida*, which is solvent-tolerant. The trans species acts like saturated fatty acids by decreasing membrane fluidity (Zhang and Rock 2008)
 Polyunsaturated C18:3 *not shown: C18:2*	Generally present in gram-positive bacteria (e.g. *Lactobacillus plantarum*)
 Branched iso-C17:0	Membrane fluidity depends on iso or anteiso forms. These lipids are usually observed in gram-positive bacteria. Combined substituents are also possible- iso-C17:1 and anteiso-C17:1
 Branched anteiso-C17:0	
 Docosahexaenoic acid (DHA) 22:6(*n*−3) *not shown: EPA 20:5(n−3)*	DHA and other long chain polyunsaturated fatty acids are unique to marine bacteria. PUFA increase membrane fluidity in cold, high pressure environments (Urakawa et al. 1998)

Typical metrics for describing membrane composition include average chain length and the unsaturated/saturated ratio. A variety of studies have reported changes in both of these metrics in response to challenge with inhibitory compounds (Lennen et al. 2011; Wu et al. 2012; Liu et al. 2013; Royce et al. 2013). Palmitic acid can by modified by either changing the length of the carbon backbone or adding a substituent such as a double bond or cyclopropane group (Table 7.2). An important note is that cyclopropane lipids are formed as an

intramembrane modification of a *cis*-unsaturated fatty acid and not from *de novo* fatty acid biosynthesis (Zhang and Rock 2008).

The available modifications to standard membrane lipids and relative distribution are dependent on the bacterial species and the environment. For example, branched lipids are a major component in the membrane of gram-positive bacteria (Cronan and Thomas 2009). Branched fatty acids can increase or decrease membrane fluidity, dependent upon the ante- or iso-conformation (Zhang and Rock 2008). The difference comes from the packing efficiency of the fatty acid within the lipid bilayer. The methyl group in the anteiso form is further away from the end, which increases the bulk. Another intriguing variable is the presence of cyclopropane fatty acids. These occur when the double bond in unsaturated lipids is methylated, such as by the *E. coli* Cfa enzyme (Grogan and Cronan 1997). Teichoic acid is a major component in the cell wall of gram-positive bacteria and provides structural rigidity to the cell wall by cross-linking peptidoglycan layers (Moat et al. 2002). Sterols increase the membrane rigidity, decreasing passive transport of lactic acid and its resulting accumulation (Vanderrest et al. 1995). Peptidoglycan is a three-dimensional mesh outside the plasma membrane and can serve as a structural barrier against osmotic pressure and toxic compounds (Heidrich et al. 2002; Typas et al. 2012). The outer layer of polymers on the surface of a bacteria cell, known as lipopolysaccharides (LPS), can also be a protection against inhibitors. The hydrophobicity and surface charge of the cell is attributed to LPS and may affect the interaction of the cell with inhibitors (Aono and Kobayashi 1997; Lee et al. 2009).

Each lipid component has an overall effect on the fluidity of the membrane through hydrophobic interactions between the lipids and proteins in the membrane (Zhang and Rock 2008). Membrane fluidity is an important part of cellular physiology and affects the function of the membrane, including respiration, passive and active transport, and protein function. Typically, as the length of the fatty acid increases the membrane fluidity decreases due to a higher packing efficiency (Mykytczuk et al. 2007). Fatty acid length has also an effect on the membrane thickness (Lewis and Engelman 1983; In't Veld et al. 1991) and curvature (Xu et al. 2008). The membrane thickness is important for proper membrane function such as protein conformation and nutrient transport (In't Veld et al. 1991; Engelman 2005). Membrane fluidity is temperature-dependent; at low temperatures, bacterial membranes are generally less fluid (Zhang and Rock 2008). *Moritella marina*, a bacterial species found in deep, cold ocean waters (Kautharapu and Jarboe 2012) copes with this by producing polyunsaturated fatty acids that then increase the fluidity of the membrane.

Membrane fluidity needs to be in an optimal range for proper membrane function. Solvents, such as alcohols and aromatics, have a fluidizing effect on the membrane (Bernal et al. 2007; Huffer et al. 2011), and thus a change in the membrane fluidity is needed to counterbalance fluidizing agents. Bacteria

respond to solvent stress in a number of ways, most notably a change in the membrane lipid composition. The ethanol producer *Zymomonas mobilis* contains relatively more octadecenoic fatty acid (C18:1) than *E. coli* (Liu and Qureshi 2009). It has been shown that *E. coli* exposure to ethanol increases the relative amount of C18:1 compared to C16:0. This change is accompanied by an altered growth rate and membrane fluidity (Ingram 1982; Liu and Qureshi 2009). This difference in membrane composition could explain why *Z. mobilis* has increased ethanol tolerance. *Bacillus cereus* predominantly uses branched lipids in its membrane; the relative abundance of saturated and iso-branched lipids increases during anaerobiosis, presumably in response to ethanol production (de Sarrau et al. 2012). Biosynthesis of teichoic acid and capsular polysaccarides by *Lactobacillus plantarum* is stimulated by the presence of ethanol (van Bokhorst-van de Veen et al. 2011), leading to the suggestion that an increase in the cell wall thickness can act as a barrier against solvents.

Cyclopropane fatty acids have been demonstrated as useful (Chang and Cronan 1999; Shabala and Ross 2008), but not essential (To et al. 2011) for microbial acid tolerance. Motivated by an observed increase in both saturated and cyclopropane fatty acids in *Clostridium acetobutylicum* during butanol challenge (Vollherbstschneck et al. 1984), Zhao et al. overexpressed the native *cfa* gene in *C. acetobutylicum*. This increased Cfa expression did enable increased butanol tolerance, though it was unfortunately accompanied by decreased butanol production (Zhao et al. 2003). It may be that a change in the membrane properties of *C. acetobutylicum* upon overexpression of *cfa* decreased solventogenesis. Liu et al. enabled cyclopropane fatty acid production in *S. cerevisiae*, an organism that does not inherently produce these compounds, in order to mitigate membrane leakage during octanoic acid challenge (Liu et al. 2013). While the cyclopropane fatty acids were successfully produced, there was no change in octanoic acid tolerance.

Luo et al. increased expression of the native *fabA* desaturase in *E. coli* with the goal of improving ethanol tolerance (Luo et al. 2009). This *fabA* overexpression increased the saturated lipid content and improved ethanol tolerance. The physiological effect of *fabA* overexpression is not clear at this time, but an increase in saturated fatty acids inherently decreases the fluidity of the membrane, possibly either affecting membrane functions or the transport of ethanol into the cell.

Similarly, Lennen and Pfleger noted a decrease in saturated fatty acid content in the cell membrane during production of carboxylic acids and proposed that this change in membrane composition was actually a mechanism of carboxylic acid toxicity. They successfully engineered their strain to restore closer to normal saturated fatty acid content, though levels were still lower than that observed for the nonproducing control strain (Lennen and Pfleger 2013). The viability of the modified carboxylic-acid producing strains was significantly

increased in the strain engineered for increased saturated fatty acid content (Lennen and Pfleger 2013). Other membrane engineering strategies inspired by microbial evolution experiments are discussed below.

7.5 Evolutionary and Metagenomic Strategies for Increasing Tolerance

Evolution is essentially a selection of random mutations that confer increased fitness (Elena and Lenski 2003; Demain 2006; Beaume et al. 2013; Kussell 2013). These mutations can take a variety of forms and influence protein function via changes in amino acid sequence or increased, decreased, or even total lack of gene expression. This evolutionary process has been leveraged in the development of strains with useful metabolic behaviors (Yomano et al. 2008; Zhang et al. 2009; Cobb et al. 2012; Reyes et al. 2013; Jin et al. 2016), but here we are more interested in its use in the development of strains with increased robustness to organic inhibitors. While these robust strains are inherently useful, identifying and understanding the mutations that confer increased robustness can enable development of strain engineering strategies. The idea that we can distill metabolic engineering strategies from the evolved strains follows the proverb commonly known as Orgel's Second Rule: "evolution is cleverer than you are." The results of reverse engineering studies are included in a distinct section below. Table 7.4 lists selected reports of evolutionary studies for ethanol, acetate, and butanol.

The most basic evolutionary studies take advantage of the natural "background" mutation rate. For wild-type *E. coli*, the most recent estimate for the rate of point mutations is 8.9×10^{-11} events per base pair per generation (Wielgoss et al. 2011). The mutation rate can be increased by the use of mutagens, such as ethyl methane sulfonate (EMS) (Kim et al. 2007), or deletion of proofreading enzymes, such as the methyl-directed mismatch repair system (Shaver et al. 2002). Such studies have successfully evolved tolerance to inhibitory compounds, such as furfural (Lin et al. 2009; Miller et al. 2009b), isobutanol (Atsumi et al. 2010; Minty et al. 2011), 2-butanol (Ghiaci et al. 2013), acetic acid (Steiner and Sauer 2003), biomass pyrolysate (Liang et al. 2013), biomass hydrolysate (Huang et al. 2009; Mills et al. 2009; Geddes et al. 2011), and others. In this traditional evolutionary scheme, cells are typically subdiluted in batch cultures on a regular basis (i.e. every 24 or 48 hours) or upon attainment of a certain cell density. Inhibitor concentration is typically increased in a stepwise fashion as cells gain tolerance. An interesting variation of this traditional approach is the Visualizing Evolution in Real Time (VERT) method, in which fluorescence-based cell sorting is used to track distinct gains in fitness (Reyes et al. 2012a; Winkler and Kao 2012; Winkler et al. 2013); the utility of this

Table 7.4 Evolution for inhibitor tolerance. It is important to note that many mutations are synergistic in development of the final tolerance phenotype.

Organism	Evolutionary method	Result	Reference
Inhibitor: Ethanol			
E. coli	SCALEs	Enriched populations in 15 and 30 g l^{-1} ethanol in minimal media	Woodruff et al. (2013)
	Transcription Machinery Engineering of Sigma 70	Final strain showed growth (6-h doubling time) in the presence of 60 g l^{-1} ethanol in rich media; control strain had no growth	Alper and Stephanopoulos (2007)
	Transcription Machinery Engineering of CRP	Final strain had a growth rate of 0.08 h^{-1} in the presence of 62 g l^{-1} ethanol, relative to 0.06 h^{-1} of original strain	Chong et al. (2013)
	Transcription Machinery Engineering of IrrE	Best strain showed a 10-fold increase in the number of cells surviving 1-h shock with 12.5% ethanol	Chen et al. (2011)
S. cerevisiae	Transcription Machinery Engineering of Spt15	Significantly improved viability during 30 h of culturing with 20% ethanol	Alper and Stephanopoulos (2007)
	Transcription Machinery Engineering of Spt15	Evolved strains grew in the presence of 15% ethanol; control strain did not tolerance concentrations above 10%	Yang et al. (2011)
Inhibitor: Acetate			
E. coli	SCALEs	Enriched population in 1.75 g l^{-1} acetate in minimal media at neutral pH	Sandoval et al. (2011)
	Transcription Machinery Engineering of IrrE	Best strain showed increased growth in the presence of 0.05% acetate in rich medium	Chen et al. (2011)
Inhibitor: Butanol			
E. coli	Sequential transfers	Increased growth and viability in the presence of 6 and 8 g l^{-1} isobutanol in rich medium	Atsumi et al. (2010)
	Sequential transfers	Increased growth in the presence of 1% (w/v) isobutanol	Minty et al. (2011)
	VERT	At least 10-fold increased survival to shock with 2 vol% *n*-butanol for 1 h in minimal medium	Reyes et al. (2012b)
	Transcription Machinery Engineering of IrrE	Best strain showed a 100-fold increase in the number of cells surviving 1-h shock with 2.1% butanol	Chen et al. (2011)

method was demonstrated during evolution of *n*-butanol tolerance in *E. coli* (Reyes et al. 2012b).

Other evolutionary-type studies rely on growth-based selection for improved tolerance, but do not only rely on the natural background mutation rate. Historically, transposon mutagenesis to randomly inactivate genes has been an effective method for identifying genes involved in a particular phenotype (Suzuki et al. 2008). The multi-SCalar Analysis of Library Enrichments (SCALEs) method uses a plasmid library containing *E. coli* genomic fragments of various lengths to comprehensively increase expression of individual genes and gene clusters (Gill et al. 2002; Lynch et al. 2007; Bonomo et al. 2008; Warner et al. 2009). DNA fragments and genes conferring increased tolerance to the focal compound are identified and interpreted. This approach has been used with ethanol (Woodruff et al. 2013), acetate (Sandoval et al. 2011), 3-HP (Warnecke et al. 2010, 2012), and 1-naphthol (Gall et al. 2008).

Another opportunity for biological discovery is the use of metagenomic screens, in which a plasmid library is generated from a mixture of various genomes and screened for the desired activity in an inhibitory condition, are most often used to find enzymes that are tolerant to specific stresses (Lu et al. 2013; Shi et al. 2013). However, a search of a DNA library generated from the human gut microbiota not only identified multiple salt-tolerant *Collinsella* genes, they also found that expression of these genes in *E. coli* resulted in increased salt tolerance (Culligan et al. 2012). These metagenomic screens provide the opportunity to discover and utilize tolerance-conferring genes from organisms or pathways that have not yet been characterized.

Expression libraries typically only enable expression of one gene or multiple genes that are co-located within the genome. It has been shown that a stepwise, combinatorial approach to expression libraries can further improve tolerance (Nicolaou et al. 2011). This approach has been demonstrated, for example, in regards to oxidative stress (Nicolaou et al. 2013), acid stress (Gaida et al. 2013), and ethanol (Nicolaou et al. 2012).

Instead of relying on altered expression of a few plasmid-associated genes, global transcription machinery engineering (gTME) uses variants of genetic regulators to perturb the expression of multiple genes (Alper and Stephanopoulos 2007; Lanza and Alper 2012). For example, ethanol tolerance in *E. coli* has been addressed by enriching library of cAMP receptor protein mutants (Chong et al. 2013), *Rhodococcus ruber* tolerance of acrylamide and acrylonirtrile through mutation of sigma 70 (Ma and Yu 2012), ethanol and biomass hydrolysate tolerance in *S. cerevisiae* through mutation of the TATA-binding protein (Liu et al. 2011; Yang et al. 2011). gTME can also be applied to a foreign regulator. Specifically, a mutation library of IrrE global regulator from radiation-resistant *Deinococcus radiodurans* has been introduced into *E. coli* and screened for resistance to a variety of inhibitors, such as ethanol,

butanol, acetate, osmotic stress, biomass hydrolysate, and salt stress (Pan et al. 2009; Zhang et al. 2010; Chen et al. 2011; Ma et al. 2011; Wang et al. 2012).

7.6 Reverse Engineering of Improved Strains

One of the major benefits of reverse engineering-evolved strains is the opportunity for biological discovery. For example, reverse engineering of furfural-tolerant *E. coli* identified YqhD as the major *E. coli* furfural reductase (Miller et al. 2009b). However, not all reverse engineering results are so clear-cut. Analysis of the results of SCALEs for 3-HP tolerance identified the 21-amino acid peptide IroK as important to 3-HP tolerance, independent of 3-HP transport, but the biological function of this peptide remains unknown at this time (Warnecke et al. 2012).

Several analyses of *E. coli* strains evolved for isobutanol or *n*-butanol tolerance have noted possible changes in LPS content or composition (Atsumi et al. 2010; Minty et al. 2011; Reyes et al. 2013). One evolved isobutanol-tolerant mutant had an insertion mutation that essentially inactivated the gene *yhbJ*, a mutation that can lead to increased production of glucosamine-6-phosphate, a major component of peptidoglycan and LPS synthesis (Atsumi et al. 2010). Another reverse engineering of isobutanol-tolerant *E. coli* found mutations in *fepE* and *yjgQ*, which contribute to LPS synthesis as well as *eptB*, which adds a phophoethanolamine group (a type of phospholipid) to LPS (Minty et al. 2011). Minty et al. found that a mutated form, designated *hfq**, can increase isobutanol tolerance (Minty et al. 2011). Hfq is an RNA-binding protein that affects RNA processing and regulation.

Each of these different evolutionary methods and subsequent reverse engineering efforts provide the opportunity to gain insight into mechanisms of inhibition and methods for increasing tolerance. These methods usually highlight multiple genes and traits that lead to a combinatorial form of tolerance. Note that it has been challenging to distill clear design strategies from some of the more advanced evolutionary schemes due to the large number of genes with perturbed expression.

It is important to note that many mutations found in evolution studies are synergistic in development of the final tolerance phenotype; rarely does one mutation dominate. It was suggested that global regulators at the transcriptional and post-transcriptional level may be key components in metabolic engineering for tolerance due to their vast reach over the genome. Interestingly, stress responses may either overlap (van Bokhorst-van de Veen et al. 2011) or be antagonistic (Reyes et al. 2013). This result is consistent with the idea that genes of overlapping function have a negative epistasis (He et al. 2010). Epistasis can be quantified by a multiplicative fitness model (Minty et al. 2011) that can analyze how particular mutations are affected by subsequent mutations.

7.7 Concluding Remarks

Production of biorenewable fuels and chemicals from biomass is desirable as a means of potentially increasing energy security, stabilizing fuels costs and addressing climate change and increasing carbon dioxide levels. Microbes are an appealing biocatalyst for this production and excellent strategies and techniques for rationally and predictably engineering biocatalyst metabolism have been developed. However, biocatalyst inhibition by either the product compound or inhibitors in the cheap, "dirty," biomass-derived sugars remain problematic in the attainment of economically viable yields, titers, and productivities. Multiple approaches can be used to increase titers and productivities: removal of the inhibitory organic compounds, redistribution of metabolic flux to limit production of inhibitory side products, and improving microbial robustness. Here we have summarized representative mechanisms of inhibition and evolutionary and engineering strategies for increasing tolerance. Evolutionary strategies have proven quite adept at increasing tolerance (Table 7.1), but additional efforts on reverse engineering these improved strains can provide guidance for new design strategies.

Acknowledgments

This work was supported by the NSF Engineering Research Center for Biorenewable Chemicals (CBiRC), NSF award number EEC-0813570.

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